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(54) COMPONENT B AS ANGIOGENIC AGENT IN COMBINATION WITH HUMAN GROWTH FACTORS

KOMPONENTE B ALS ANGIOGENES MITTEL ZUSAMMEN MIT HUMANEN WACHSTUMSFAKTOREN

COMPOSE B EN TANT QU'AGENT ANGIOGENIQUE COMBINE A DES FACTEURS DE CROISSANCE HUMAINE

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(73) Proprietor: Applied Research Systems ARS Holding N.V.
Curacao (AN)

(72) Inventors:
- ZICHE, Marina
I-50144 Firenze (IT)
- DONINI, Silvia
I-00186 Roma (IT)
- BORRELLI, Francesco
I-00179 Roma (IT)

(74) Representative: Gervasi, Gemma, Dr.
NOTARBARTOLO & GERVASI Srl,
Corso di Porta Vittoria, 9
20122 Milano (IT)

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- J. FOLKMAN: "CLINICAL APPLICATIONS OF RESEARCH ON ANGIOGENESIS" THE NEW ENGLAND JOURNAL OF MEDICINE, vol. 333, no. 26, 28 December 1995 (1995-12-28), pages 1757-1763, XP000654557 BOSTON, MA, US

EP 1 093 380 B1

Description

Field of the invention

[0001] The present invention refers to the use of Component B as angiogenic agent in combination with human growth factors.

State of the art

[0002] Component B (hereinafter indicated as CB) is an 81 amino acid protein originally isolated from human urine. The human gene expressing the protein has been cloned and expressed in CHO cells as recombinant human Component B, the protein has a molecular weight of about 8.9 kD and was thoroughly described in WO 94/14959 to which reference is made also for the methods of preparation and its amino acid sequence.

[0003] In WO97/39765 the use of CB as cicatrizant was described.

[0004] It is also known that growth factors, as for example basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF) have angiogenic activity.

Brief description of the drawings

[0005] Figure 1: Shows the implant of a pellet loaded with both active compounds (1A) or of two pellets each loaded with one active compound (1B) in the rabbit cornea.

[0006] Figure 2: Shows the effect of bFGF and CB on angiogenesis in the rabbit cornea.

[0007] Figure 3: Shows the effect of heat-inactivation on the angiogenic activity of CB.

[0008] Figure 4: Shows the synergistic effect of CB on bFGF-induced angiogenesis.

[0009] Figure 5: Shows the synergistic effect of CB on the angiogenic activity of bFGF.

[0010] Figure 6: Represents a drawing of a typical histological section of rabbit cornea illustrating the main structures observable in following figures 7 - 11.

[0011] Figure 7: section of rabbit cornea (x200) with a corneal pocket containing 100 ng of bFGF sampled at 6 days post-surgery wherein the arrows show neoformed vessels. (Ep = epithelium).

[0012] Figure 8: section of rabbit cornea (x100) with a corneal pocket containing 500 ng of CB sampled at 2 days post-surgery wherein the arrows show neoformed vessels.

[0013] Figure 9: section of rabbit cornea (x 200) with a corneal pocket containing 500 ng of CB sampled at 6 days post-surgery wherein the arrow shows neoformed vessels.

[0014] Figure 10: Section of rabbit cornea (x100) with a corneal pocket containing 4 µg of CB sampled at 15 days post-surgery wherein the arrows show neoformed vessels. Figure 11: Section of rabbit cornea (x100) with a corneal pocket containing 500 ng of bFGF sampled at 15 days post-surgery wherein the arrow shows neoformed vessels.

[0015] Figure 12: mean and 95.0% Tukey HSD Intervals

Detailed description of the invention

[0016] It has now surprisingly been found that the simultaneous presence of CB and a growth factor increases the angiogenic response elicited by either individual agent, in other words CB is capable of synergizing with a growth factor in promoting neovascular growth in tissues probably facilitating some early events required to mobilise endothelial cells from capillaries.

[0017] An object of the present invention is to provide for the use of CB in combination with a growth factor for the manufacture of pharmaceutical compositions useful for the treatment of wounds, ulcers and other traumatic lesions to any tissues in the body.

[0018] Another object of the invention is a pharmaceutical composition prepared as described above.

[0019] The administration of the active ingredient may be oral, intravenous, intramuscular, subcutaneous or topical. Other routes of administration, which may establish the desired blood levels of respective active agents are comprised by the present invention.

[0020] The administration of the two active compounds can be performed by a single pharmaceutical preparation containing both of them or, preferably, by two pharmaceutical preparations each containing separately one of the two ingredients.

[0021] Preferred growth factors to be used in combination with CB according to the invention are basic fibroblast growth factor (bFGF) or vascular endothelial growth factor (VEGF).

[0022] Angiogenesis was studied in the cornea of albino rabbits since this is an avascular and transparent tissue where inflammatory reactions and growing capillaries can be easily monitored and changes quantified by stereomi-

croscopic examination (Ziche et al., 1982). This method allows the monitoring over an extended period of time of vessel growth by direct and non traumatic observation of the process. Moreover in the same animal the quantification of the effect can be compared with that of a known agent.

[0023] The investigation of the role of Component B (CB) in *in vivo* angiogenesis studied in the rabbit cornea assay was performed by:

a) testing the ability of the molecule to produce vessel growth when placed into the avascular corneal stroma;
b) testing the ability of the molecule to favour or repress neovascularization elicited by the angiogenesis factor basic fibroblast growth factor (bFGF) or vascular endotelial growth factor (VEGF).

Methods

Protocol for slow-release preparation of test compounds

[0024] Growth factors or peptides were prepared as slow-release pellets.

[0025] Slow-release pellets (1x1x0.5 mm) were prepared in sterile conditions incorporating the test substances into a casting solution of a ethinyl-vinyl copolymer (Elvax-40, Dupont, Wilmington, Delaware), in 10% methylene chloride (10 μl/droplet) (Langer and Folkman, 1976; Ziche et al. 1982).

Surgical procedure

[0026] The angiogenic activity was assayed *in vivo* using the rabbit cornea assay.

[0027] In the lower half of New Zealand white rabbit eye (Charles River, Calco, Lecco,

[0028] Italy), anaesthetised with sodium pentothal (30 mg/kg), a micro pocket (1.5x3mm) was surgically produced using a pliable iris spatula 1.5 mm wide.

[0029] The pellets were implanted in the micropockets located into the transparent avascular corneal stroma.

[0030] Quantification of corneal angiogenesis Subsequent daily observations of the implants were made with a slit lamp stereomicroscope without anaesthesia. An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 3-4 days and capillaries progressed to reach the implanted pellet according to the scheme previously reported (Ziche et al. 1989). Angiogenic activity is expressed as the number of implants exhibiting neovascularization over the total implants studied. Potency is scored by the number of newly formed vessels and by their growth rate. Data are expressed as angiogenesis score, calculated as vessel density x distance from limbus in mm. A density value of 1 corresponded to 0 to 25 vessels per cornea, 2 from 25 to 50, 3 from 50 to 75, 4 from 75 to 100 and 5 for more than 100 vessels (Ziche et al., 1994).

Experimental design

[0031] The effect of Component B was tested following two procedures:

A) Three different concentrations of the molecule were tested in the cornea of at least 4 distinct rabbits per each dose, to define the potential angiogenic activity of the compound. The effect of Component B was compared with that elicited by the growth factor bFGF at 50 and 100 nag/pellet. In this experimental protocol rabbits were monitored for 3 weeks.
B) To evaluate a potential role of Component B in modulating angiogenesis the effect of this agent was tested in the presence of a defined angiogenesis factor, i.e. bFGF. To this aim two adjacent pockets were surgically produced in the same cornea, one bearing the angiogenic trigger and the other Component B. Experiments were also performed testing both substances incorporated into the same pellet (Figure 1).

[0032] This last experimental protocol was specifically set up by our group to define:

1) the effect of the agent as a "costimulator" of the angiogenesis elicited by bFGF; or 2) the ability of the agent to inhibit angiogenesis elicited by the growth factor (Ziche et al, 1992 and 1994).

[0033] In this experimental protocol the rabbits were monitored for 4-5 weeks. The same protocol was used to test the effect of CB as "costimulator" of angiogenesis induced by VEGF.

Histological analysis

[0034] Rabbit corneas with corneal pockets containing CB and/or bFGF were sampled at 2, 6, 15 days post surgery, and fixed in formalin after removing the pellets.
[0035] Routine histopathological processing was performed; sections 5μm thick were cut next to where each pellet was placed; sections were stained with hematoxylineosin. At least 40 sections were examined per each cornea.

Statistical analysis

[0036] Results are expressed as means for (n) implants. Angiogenic score data contained both positive and negative results. Multiple comparisons were performed by one-way ANOVA and individual differences were tested by Fisher's test after the demonstration of significant intergroup differences by ANOVA. A P value <0.05 was taken as significant (see also Appendix for further statistical evaluation).

Results

a) Angiogenic activity of CB

[0037] The angiogenic activity of CB was tested after incorporating increasing concentrations of the compound in slow release pellets of the polymer Elvax-40. Solubilization and incorporation of the compound into the polymer pellets did not cause any specific problem. The doses tested were: 0.2, 0.5, 2 and 4 μg/pellet. The effect of CB was compared to that produced by basic fibroblast growth factor (bFGF).
[0038] CB elicited a dose-dependent angiogenic effect that appeared to be weaker than the one elicited by bFGF. In Figure 2A data are reported on the angiogenic activity of bFGF obtained from previous experiments and from the experiments run in parallel with CB. In Figure 2B data from daily observation of rabbit corneas implanted with CB-containing pellets are reported as angiogenic score. The highest angiogenic score obtained with CB averaged around 3-3.5 (2-4 μg/pellet) (P<0.05 vs vehicle pellets alone) vs 7-8 produced by bFGF (0.2 μg/pellet)(P<0.05 vs vehicle pellets alone). CB was not angiogenic at the concentration of 0.2 μg/pellet. As shown in Table 1, 0.5 μg/pellet CB induced a positive angiogenic response in 1 implant out of 5 performed. Two and 4 μg/pellet were the most effective doses. These doses induced a similar angiogenic activity and produced 2 positive implants out of 5 performed.
[0039] CB was devoid of any macroscopic inflammatory activity as revealed by the persistence of corneal transparency all through the experiments at any concentration tested.
[0040] To assess the specificity of CB angiogenic effect, the compound was heat-inactivated (h.i.) by boiling it for 20 min. The dose of 2 μg was then tested.
[0041] Following heat inactivation CB completely lost angiogenic activity (Fig. 3)(P<0.05 vs CB 2μg).

b) Effect of CB on the angiogenesis induced by bFGF

[0042] To evaluate the potential role of CB in modulating the effect of a known angiogenic effector, experiments were performed testing suboptimal concentrations of both substances (500 ng of CB and 100 ng of bFGF) coreleased into the corneal stroma. Experiments were performed testing both substances incorporated into the same pellet (Fig. 1A). Furthermore the compounds were tested at the same concentration as above but released into the stroma separately in 2 independent pellets (Fig. 1B).
[0043] The simultaneous presence of CB and bFGF into the cornea increased the angiogenic response elicited by either individual agent (Fig. 4A and B, Table 2).
[0044] Angiogenesis occurred earlier and progressed more rapidly producing a significant increment of the number of newly formed vessels (P<0.05 *vs* CB and bFGF alone). This effect was apparent in both experimental conditions.
[0045] However, when CB and bFGF were released independently by 2 separate pellets the effect was higher. Capillaries grew toward bFGF rather than CB suggesting that CB contributed to potentiate bFGF activity. After 7 days, neovascular growth started to regress.
[0046] Additional experiments were performed with increasing concentrations of CB (0.2, 0.5 and 2 μg/pellet) on the angiogenesis elicited by a constant concentration of bFGF (100 ng). A synergism between the two molecules could be observed (Fig. 5). Interestingly, the most effective condition of synergism between CB and bFGF was observed with 200 ng CB (P<0.05 vs CB and bFGF alone) tested in two separate pellets.

c) Effect of CB on the angiogenesis induced by VEGF

[0047] In Table 3 the synergistic effect of Component B on VEGF-induced angiogenesis is reported.

[0048] The synergism between CB and VEGF was evaluated with the factors tested in two separate pellets. The results obtained at day 10 are reported in Table 3. The data are expressed as the number of implants exhibiting neovascularization with an angiogenesis score equal or over to 6, over the total implants performed.

[0049] Further statistical analysis was performed in order to confirm possible positive interactions between the test compounds using a more conservative analysis (see Fig. 11 in Appendix).

[0050] The main factors ("test compounds" and "angiogenic score" over time) were analysed according to the Multifactor Analysis. The results showed that statistically significant differences ($p<0.0001$) are present among the test compounds over time.

[0051] As to the interaction among the test compounds the results of the Tukey's test allow the following considerations (see Fig. 12 below):

CB 500 ng + bFGF 100 ng (1 pellet)

[0052] Both compounds individually are not statistically different from controls. The combination of the two compounds gave a response which is statistically different from either the controls and the single drugs. The response is around the expected additive effect.

CB 200 ng + bFGF 100 ng (2 pellets)

[0053] Both compounds individually are not statistically different from controls. The. combination of the two compounds gave a response which is statistically different from either the controls and the single drugs. In addition, it should be noted that the response of the combined treatments clearly exceeds the expected additive effect. The above seems to confirm the presence of a synergistic effect between the two drugs.

CB 500 ng + bFGF 100 ng (2 pellets)

[0054] Both compounds are not statistically different from controls. The combination of the two compounds gave a response which is statistically different either from the controls and the single drugs. The response is around the expected additive effect. In addition, no differences were found comparing CB 500 ng + bFGF 100ng (1 pellet) vs CB 500 ng + bFGF 100ng (2 pellet).

CB 2 μg + bFGF 100 ng (2 pellets)

[0055] Both compounds are not statistically different from controls. The combination of the two compounds gave a response which is statistically different from controls but not from the single compounds. The response is around the expected additive effect.

Histological analysis

[0056] The effect of CB was examined at the maximal effective concentration (4 μg) and at a suboptimal concentration (500 ng) in the presence and in the absence of bFGF (100 ng). No difference in the extent of cellular infiltrate was apparent between CB and bFGF implants in any combination (Figs. 7,8,9). Within 2 days from the implants a leukocyte infiltrate surrounded a dense network of newly formed capillaries in proximity of the limbal region at the epithelial side of the cornea (Fig. 10). At day 6 a consistent reduction in the extent of the leukocyte infiltrate was apparent while capillary vessels appeared increased in number and caliber in response to either molecule (Fig. 11). At day 15 the extent of the leukocyte infiltrate was negligible while capillaries appeared morphologically unmodified.

Conclusions

[0057] Component B possesses angiogenic activity which is apparent in the concentration range of micrograms and which is lost by heat inactivation. Most of the angiogenesis factors are angiogenic at concentrations 20-40 fold lower. Together with the high concentration required to elicit angiogenesis, 2 aspects appear relevant in CB effect:

1) the ability to elicit budding of capillaries within the first 3-4 days from the implant, mimicking the secreted ang-

iogenesis factor VEGF rather than the matrix linked angiogenesis factor bFGF;
2) the flattening over time of the efficiency of neovascular growth, leading to only 30-40% of the implant tested to be fully vascularized after 10-14 days.

[0058] Our results indicate that CB induces angiogenesis *in vivo* and has the ability to synergize with bFGF in promoting neovascular growth in the rabbit cornea.

[0059] These considerations together with the characteristics of the potentiation of the angiogenic response in the presence of bFGF, suggest that CB requires the presence of additional growth factors to fully express its angiogenic potential *in vivo*.

[0060] Histological examination of corneal sections sampled at various time intervals was performed to assess whether the angiogenesis process elicited by CB involved inflammatory cell infiltrate. The effect of CB was compared to that produced by the corneal implant of bFGF. At routine histological examination we did not find major differences in the extent and in the type of leukocyte infiltrate in corneas receiving CB, bFGF or the combination of the two. Thus from our results we can conclude that the corneal vascularization induced by CB does not appear to be mediated by gross inflammatory reaction products since no sign of corneal opacity was apparent.

[0061] The characteristic of the angiogenic response elicited in the avascular cornea by CB suggests that CB might facilitate some of the early events required to mobilise endothelial cells from capillaries. Once this process is started and endothelial cells are "loosened" from the tight boundary to the extracellular matrix, bFGF expresses its mitogenic effect with more efficiency.

Table 1

Effect of Component B on angiogenesis in the rabbit cornea			
CB (μg/pellet)	**Positive implants/total performed**	**bFGF (ng/pellet)**	**Positive implants/total performed**
0	0/6	0	0/6
0.2	0/5	50	1/6
0.5	1/5	100	2/6
2	2/5	200	5/6
4	2/5		

Data are expressed as positive implants exhibiting neovascularization over the total implants performed. The results obtained at day 7 are reported.

An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 3-4 days and capillaries progressed to reach the pellet containing the angiogenic factors.

Table 2

Synergistic effect of Component B on bFGF-induced angiogenesis	
Test compounds	**Positive implants/total performed**
CB 500 ng	1/5
bFGF 100 ng	1/5
CB 500 ng + bFGF 100 ng (one pellet)	3/6
CB 500 ng + bFGF 100 ng (two pellets)	4/5

Data are expressed as positive implants exhibiting neovascularization over the total implants performed. The results obtained at day 7 are reported.

An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred after 3-4 days and capillaries progressed to reach the pellet containing the angiogenic factors.

TABLE 3

Synergistic effect of Component B on VEGF-induces angiogenesis	
	Positive implants/total performed
CB 200 ng	0/5

TABLE 3 (continued)

	Positive implants/total performed
CB 500 ng	1/5
VEGF 100 ng	1/4
CB 200 ng + VEGF 100 ng	2/4
CB 400 ng + VEGF 100 ng	4/4

Claims

1. Use of Component B in combination with a human growth factor for the preparation of a composition for promoting angiogenesis.

2. The use according to Claim 1 in the treatment of wounds, ulcers and other traumatic lesions to any of the tissues in the body.

3. Pharmaceutical composition useful as cicatrizants comprising Component B and a human growth factor as active principles in combination with a pharmaceutically acceptable carrier.

4. Pharmaceutical composition according to Claim 3 wherein the two active principle are both present in a single administration dose.

5. Pharmaceutical composition according to Claim 3 wherein the two active principle are present each in a separated administration dose.

6. Pharmaceutical composition according to Claims 3 - 5 wherein the human growth factor is bFGF or VEGF.

7. Use of Component B, in combination with a human growth factor, for the preparation of a pharmaceutical composition for the treatment of wounds, ulcers and other traumatic lesions to any of the tissues in the body.

Patentansprüche

1. Verwendung der Komponente B zusammen mit einem humanen Wachstumsfaktor zur Herstellung einer Zusammensetzung zur Förderung der Angiogenese.

2. Die Verwendung gemäß Anspruch 1 zur Behandlung von Wunden, Geschwüren und anderen traumatischen Verletzungen von jeglichen Körpergeweben.

3. Pharmazeutische Zusammensetzung zur Verwendung als Narbenbildung-fördemdes Mittel enthaltend Komponente B und einen humanen Wachstumsfaktor als wirksame Mittel zusammen mit einem pharmazeutisch verträglichen Träger.

4. Pharmazeutische Zusammensetzung gemäß Anspruch 3, worin die beiden wirksamen Mittel in einer gemeinsamen Dosis zur Verabreichung vorliegen.

5. Pharmazeutische Zusammensetzung gemäß Anspruch 3, worin die beiden wirksamen Mittel jeweils einzeln in getrennten Dosen zur Verabreichung vorliegen.

6. Pharmazeutische Zusammensetzung gemäß einem der Ansprüche 3 bis 5, worin der humane Wachstumsfaktor bFGF oder VEGF ist.

7. Verwendung der Komponente B zusammen mit einem humanen Wachstumsfaktor für die Herstellung einer pharmazeutischen Zusammensetzung zur Behandlung von Wunden, Geschwüren und anderen traumatischen Verletzungen von jeglichen Körpergeweben.

Revendications

1. Utilisation de composant B en combinaison avec un facteur de croissance humain pour la préparation d'une composition destinée à favoriser l'angiogenèse.

2. Utilisation selon la revendication 1, dans le traitement de plaies, d'ulcères et autres lésions traumatiques à l'un quelconque des tissus dans le corps.

3. Composition pharmaceutique utile en tant que cicatrisants comprenant le composant B et un facteur de croissance humain en tant que principes actifs en combinaison avec un véhicule acceptable sur le plan pharmaceutique.

4. Composition pharmaceutique selon la revendication 3, dans laquelle les deux principes actifs sont tous deux présents dans une dose à prise unique.

5. Composition pharmaceutique selon la revendication 3, dans laquelle les deux principes actifs sont chacun présents dans une dose à prise séparée.

6. Composition pharmaceutique selon les revendications 3 à 5, dans laquelle le facteur de croissance humain est bFGF ou VEGF.

7. Utilisation de composant B, en combinaison avec un facteur de croissance humain, pour la préparation d'une composition pharmaceutique destinée au traitement de plaies, d'ulcères et autres lésions traumatiques à l'un quelconque des tissus dans le corps.

Fig.1

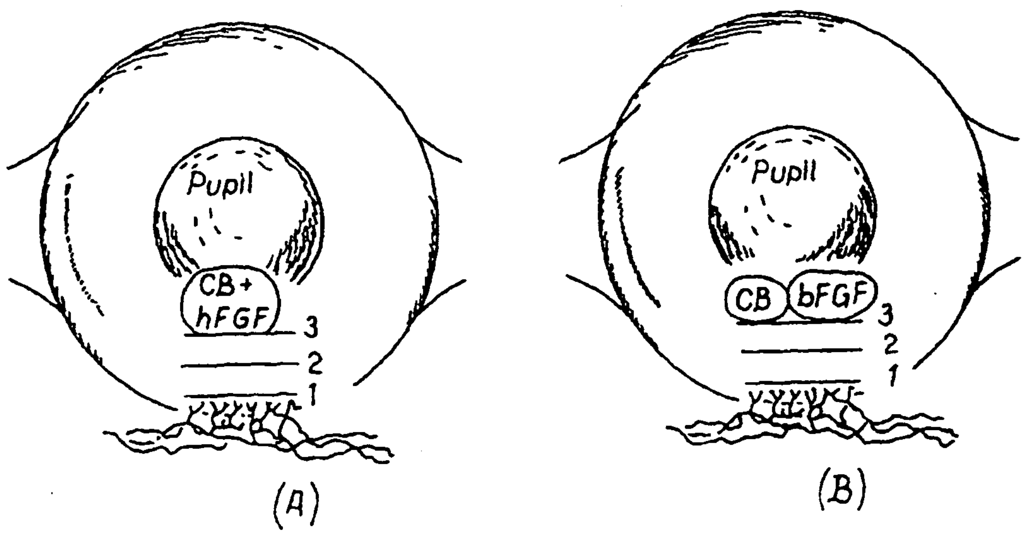

Pupil
CB+
hFGF
3
2
1
(A)
Pupil
CB
bFGF
3
2
1
(B)

Fig.2

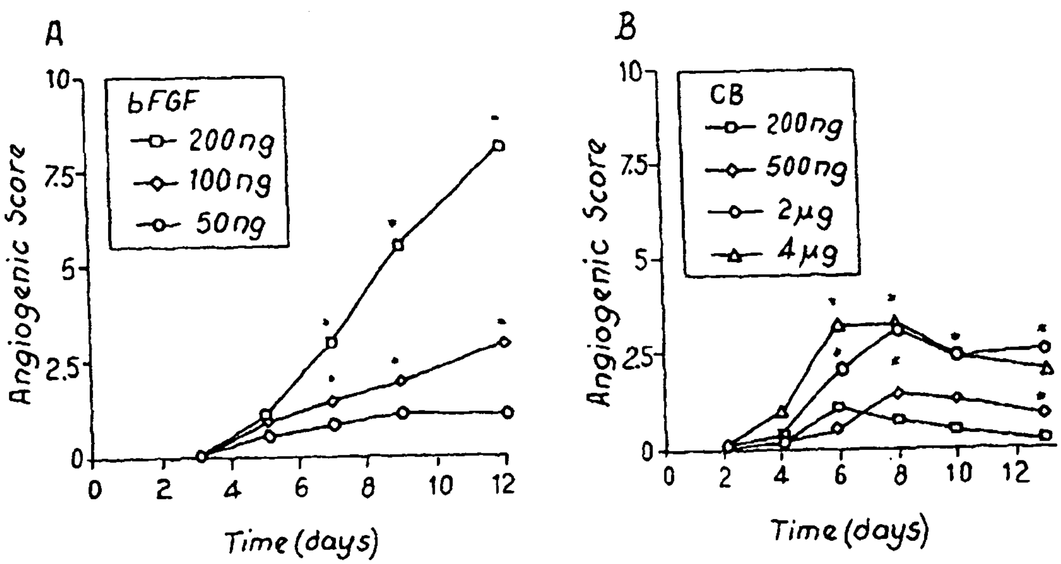

A
bFGF
200ng
100ng
50ng
Angiogenic Score
Time (days)
B
CB
200ng
500ng
2μg
4μg
Angiogenic Score
Time (days)

Fig.3

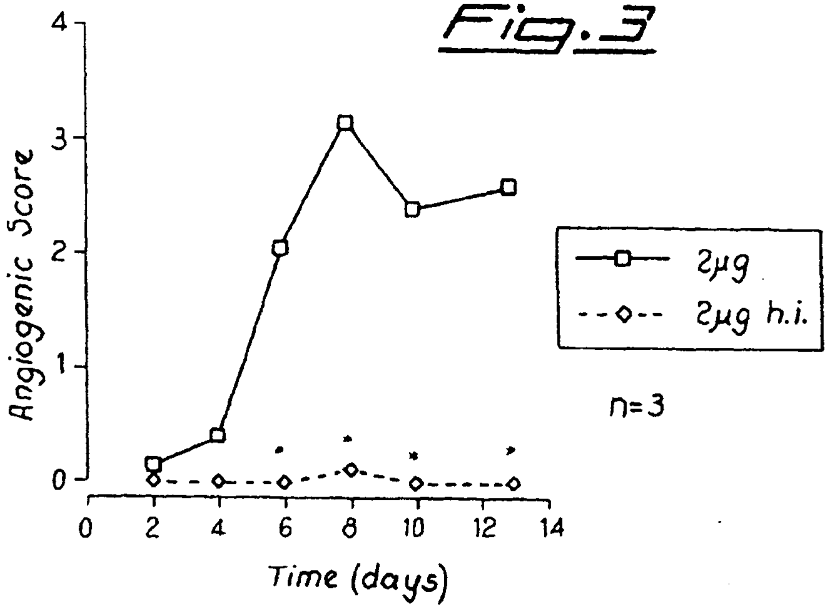
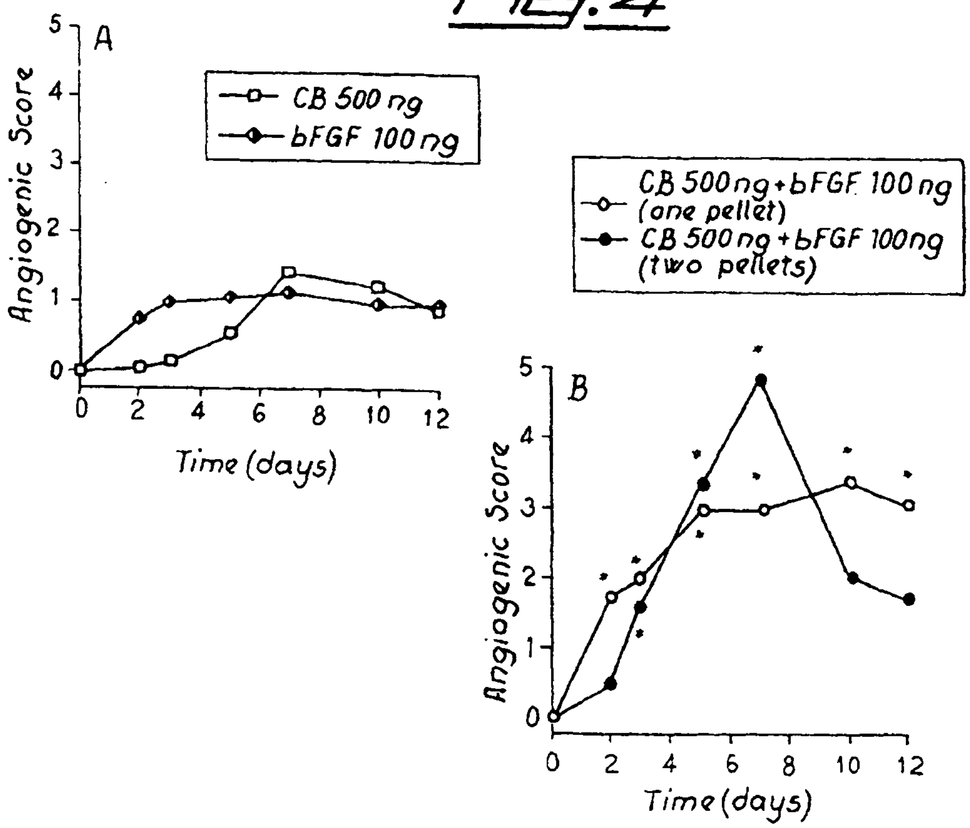

Angiogenic Score
Time (days)
2µg
2µg h.i.
n=3

Fig.4


A
Angiogenic Score
Time (days)
CB 500 ng
bFGF 100 ng
CB 500 ng+bFGF 100 ng (one pellet)
CB 500 ng+bFGF 100 ng (two pellets)
B
Angiogenic Score
Time (days)

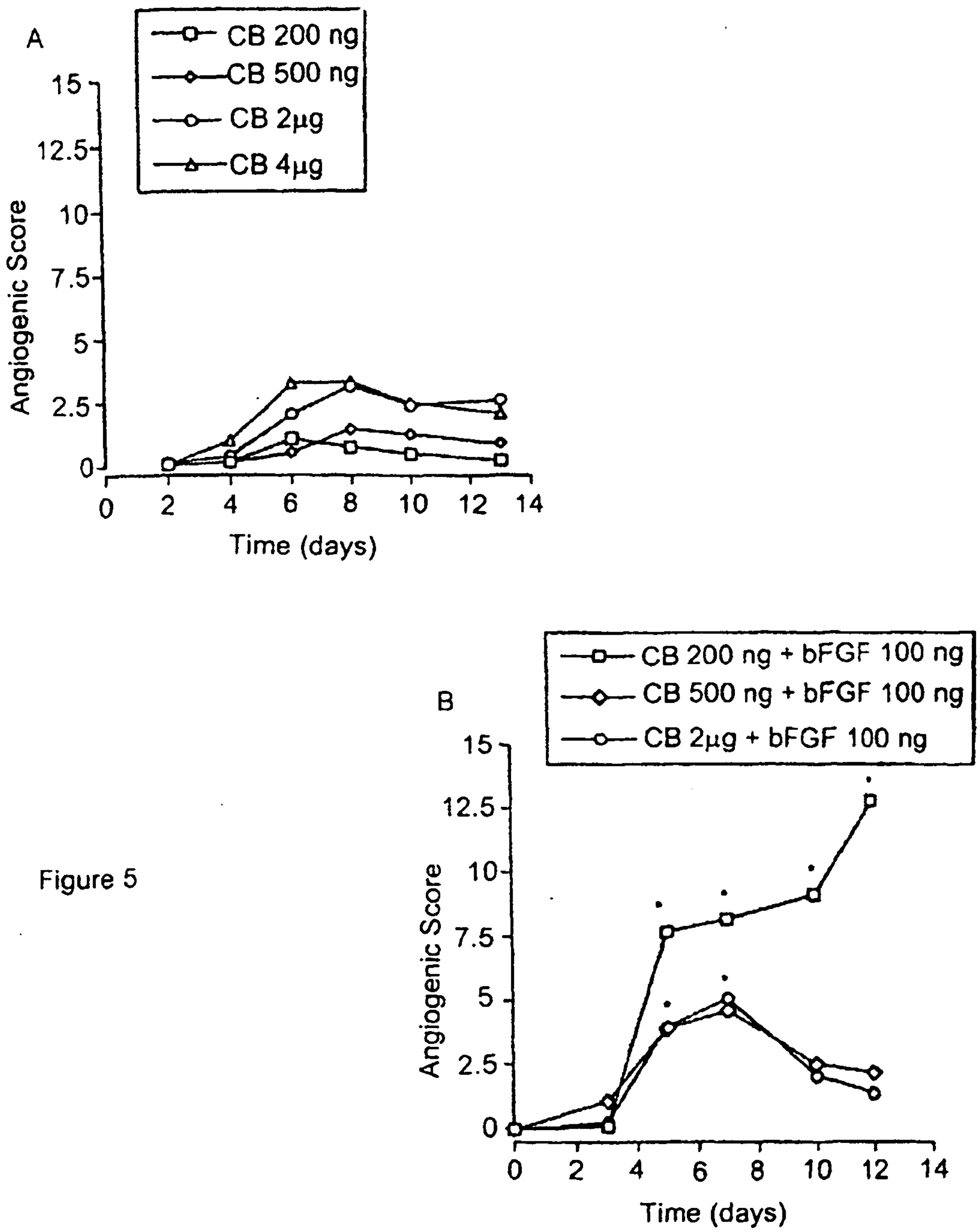

A
CB 200 ng
CB 500 ng
CB 2μg
CB 4μg
Angiogenic Score
15
12.5
10
7.5
5
2.5
0
0 2 4 6 8 10 12 14
Time (days)
B
CB 200 ng + bFGF 100 ng
CB 500 ng + bFGF 100 ng
CB 2μg + bFGF 100 ng
Angiogenic Score
15
12.5
10
7.5
5
2.5
0
0 2 4 6 8 10 12 14
Time (days)

Figure 5

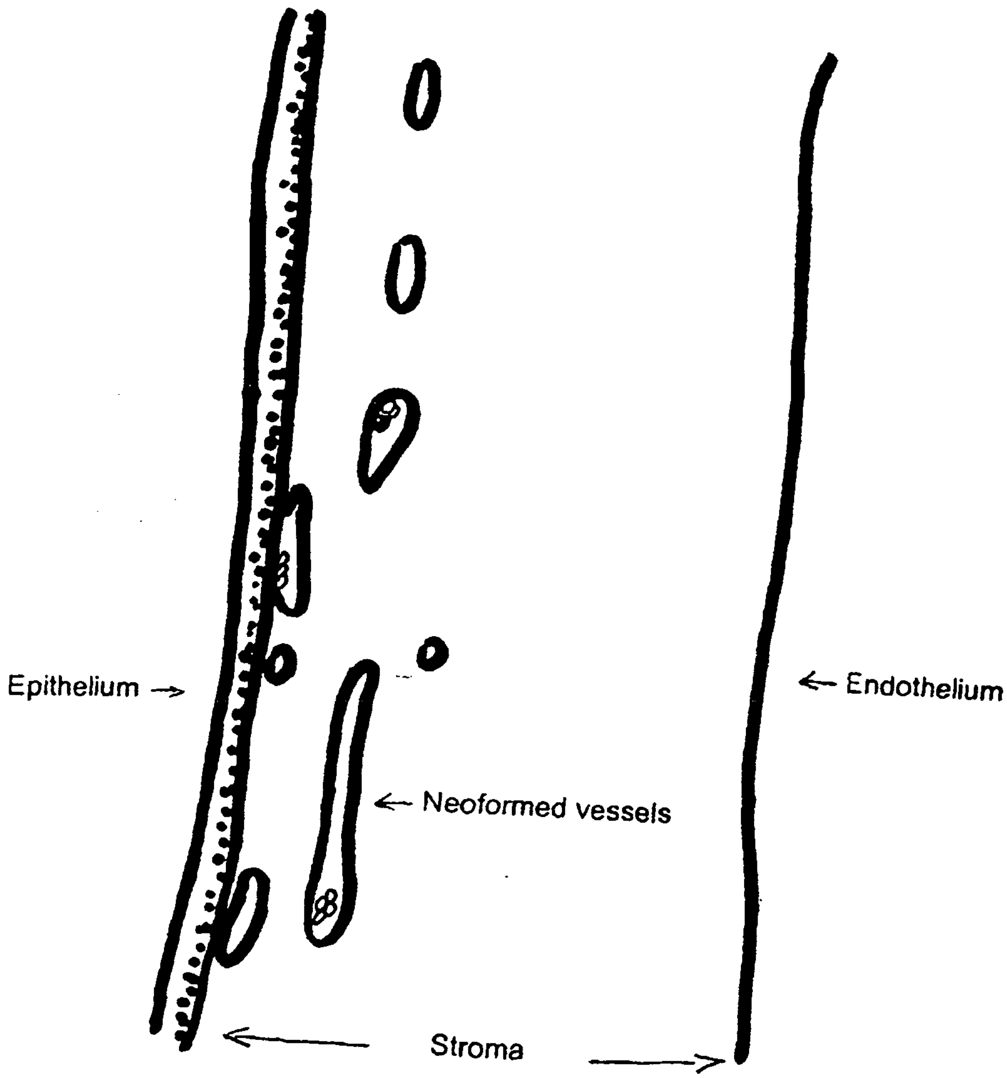


Figure 6

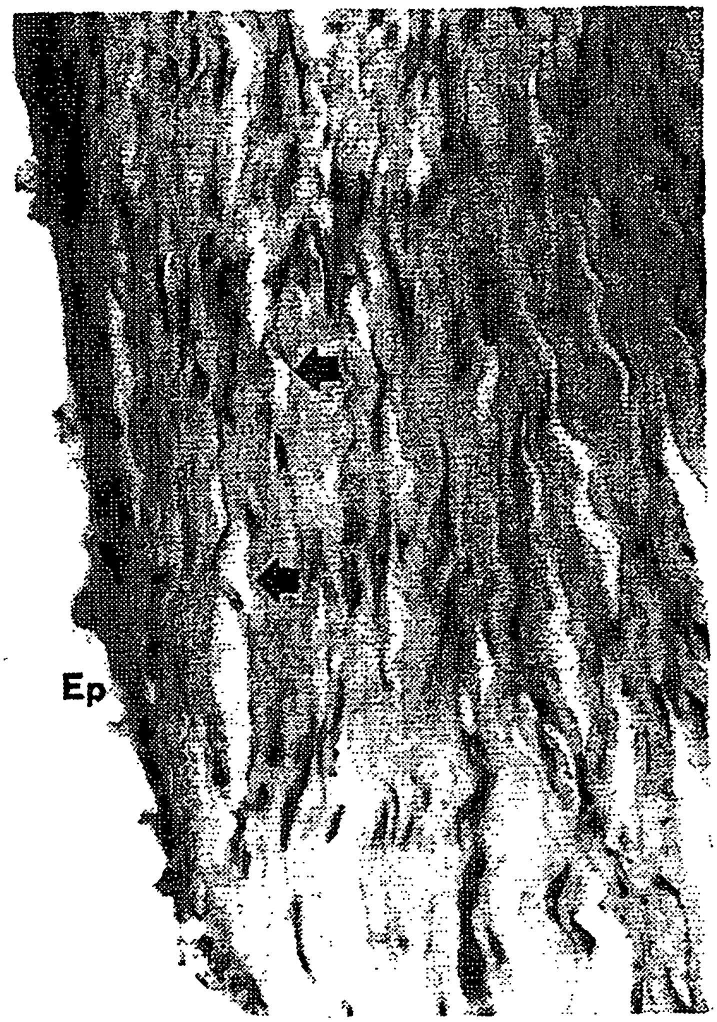


Figure 7

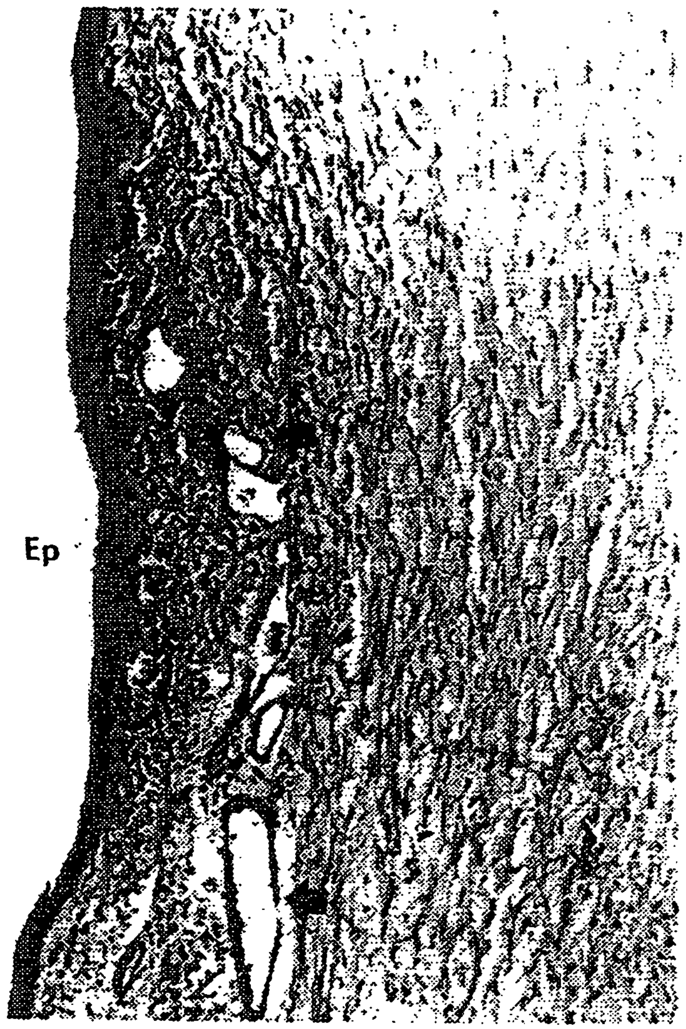


Figure 8

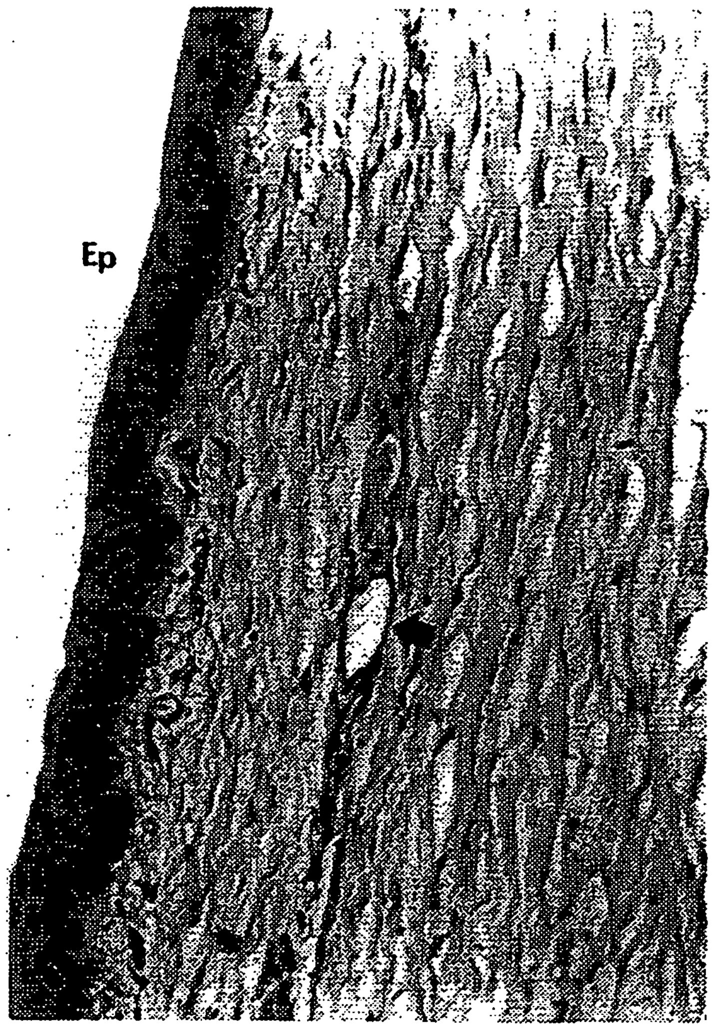


Figure 9

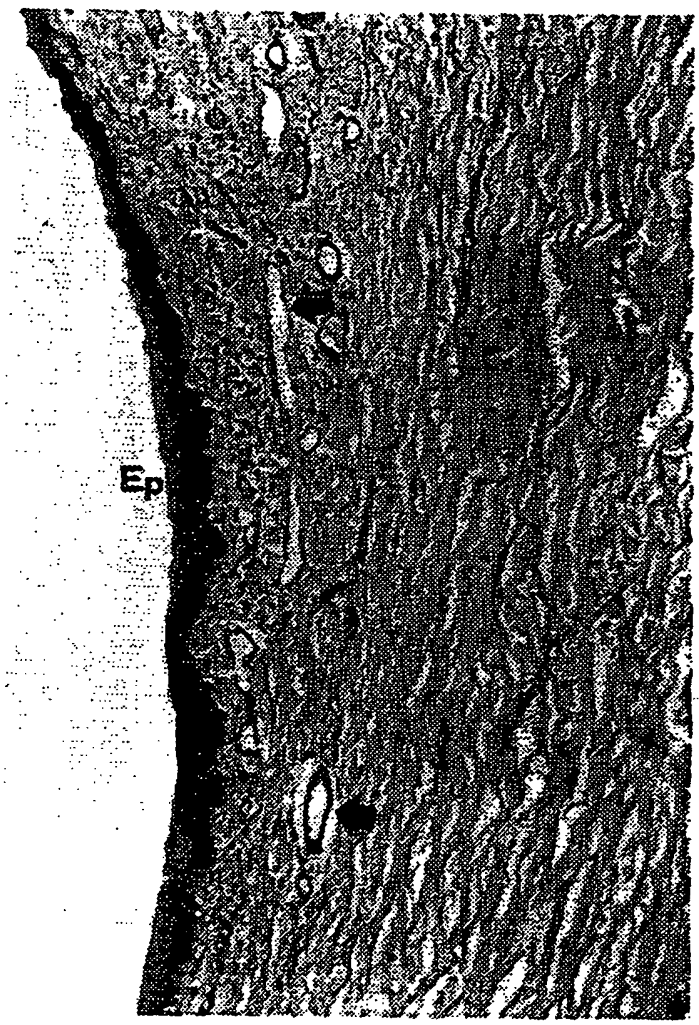


Figure 10

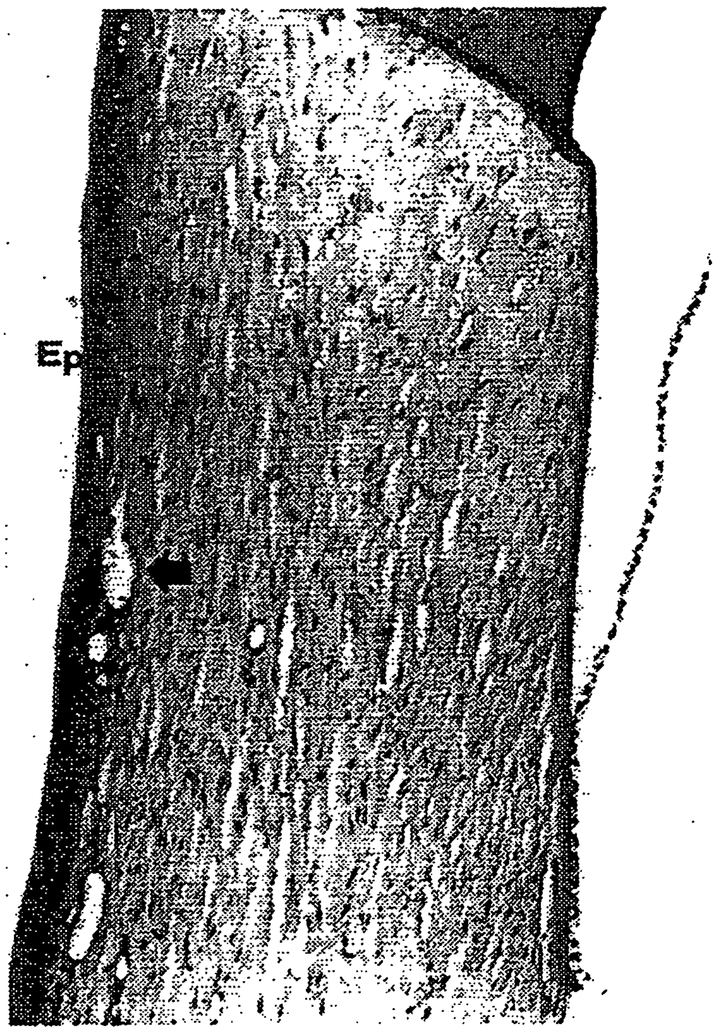
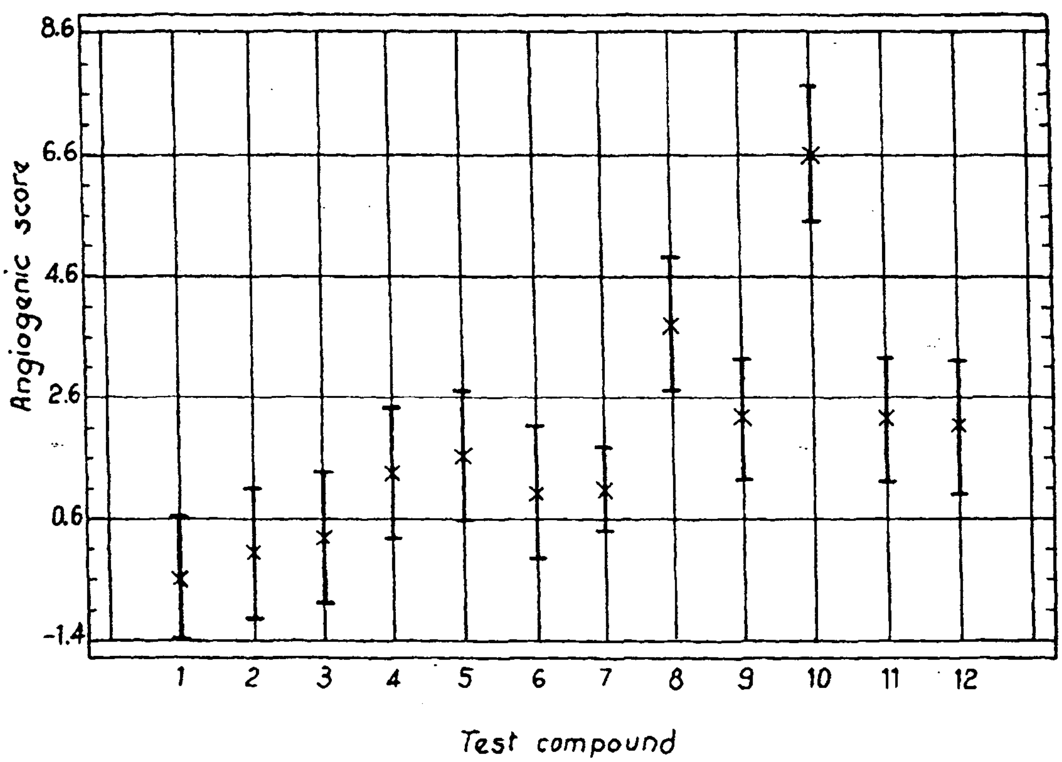


Figure 11

Fig.12


8.6
6.6
4.6
2.6
0.6
-1.4
Angiogenic score
1
2
3
4
5
6
7
8
9
10
11
12
Test compound